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1    **Role of mTOR in autophagic and lysosomal reactions to**  
2    **environmental stressors in molluscs**

3

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25

## 26    **Abstract**

27    Lysosomal membrane stability (LMS) has been used in various organisms as a very sensitive  
28    biomarker of stress. However, despite the abundance of data about regulation of the autophagic  
29    process in mammals, in the invertebrates there is only limited mechanistic understanding. Marine  
30    mussels (*Mytilus galloprovincialis* Lam.) are bivalve molluscs, widely used as models in  
31    ecotoxicology and as environmental bioindicators of sea water quality. In order to elucidate this  
32    fundamental process, in the present study, mussels were exposed for 3 days to a “priority”,  
33    ubiquitous environmental contaminant, benzo[*a*]pyrene (B[*a*]P) at different concentrations (i.e. 5,  
34    50, 100 µg/L seawater). B[*a*]P accumulated in lysosomes of digestive tubule epithelial cells  
35    (digestive cells) and in enlarged lipid-rich lysosomes (autolysosomes) as detected by  
36    immunofluorescence and UV-fluorescence. B[*a*]P also activated the autophagic process with a  
37    marked decrease of LMS and concurrent increase in lysosomal/cytoplasmic volume ratio.  
38    Dephosphorylation of mTOR contributes to increased lysosomal membrane permeability and  
39    induced autophagy. B[*a*]P induced a decrease in phosphorylated (active form) mTOR. The probable  
40    role of mTOR in cell signalling and the regulation of the cellular responses to the contaminants has  
41    been also confirmed in a field study, where there was significant inactivation of mTOR in stressed  
42    animals. Statistical and network modelling supported the empirical investigations of autophagy and  
43    mTOR; and was used to integrate the mechanistic biomarker data with chemical analysis and DNA  
44    damage.

45

46    Keywords: mussel, autophagy, B[*a*]P, mTOR, network modelling

47

## 48    **1. Introduction**

49    Over the last decades, the studies about the biological effects of environmental stressors including  
50    toxic chemicals have led to development of numerous biomarkers at different levels of functional  
51    complexity suitable to follow the evolution of the stress syndrome from the early warning signals at  
52    the molecular/cellular level to the deterioration at the organism level (Moore et al., 2012; Viarengo  
53    et al., 2007). Among others, numerous lysosomal-related biomarkers have been developed;  
54    lysosomes, highly conserved organelles playing a pivotal role in many cellular processes, were  
55    shown to be the target for a wide range of contaminants (Appelqvist et al., 2013; Moore, 1988;  
56    Moore et al., 2007; Viarengo and Nott, 1993). In particular, lysosomal membrane stability (LMS),  
57    whose reduction represents a subcellular pathological reaction known to be linked to augmented  
58    autophagic sequestration of cellular components, has been used both in invertebrates and  
59    vertebrates as a very sensitive and easy to use biomarker of stress (Fernández et al., 2005; Moore et  
60    al., 2004a; Sforzini et al., 2015; Svendsen et al., 2004).

61    Autophagy (i.e. macroautophagy), the major inducible pathway for general turnover of cytoplasmic  
62    components, takes place in all eukaryotic cells (Klionsky and Emr, 2000). This process plays an  
63    essential role in promoting cell survival in response to metabolic as well toxic stress by the  
64    sequestration of cytoplasmic components, the removal of damaged organelles and protein  
65    aggregates and their subsequent degradation in lysosomes. However, an excessive autophagic rate  
66    has been shown to have deleterious consequences for tissue/organism health (Levine and Kroemer,  
67    2008). Autophagy is well documented in marine mussels using biochemical, cell fractionation,  
68    cytochemical and ultrastructural methods, where it is induced by many environmental stressors  
69    including fasting, increased salinity, polycyclic aromatic hydrocarbons (PAHs) and chloroquine  
70    (Bayne et al., 1980; Moore, 2004, 2008; Moore & Clarke, 1982; Moore et al., 1980, 1996, 2006a, b;  
71    2007; Nott et al., 1985; Pipe & Moore, 1985).

72 Stress-induced autophagy, such as that induced by nutrient starvation, is regulated by the inhibition  
73 of mTOR (mechanistic Target of Rapamycin) in eukaryotic cells from yeast to mammals (Klionsky  
74 and Emr, 2000; Moore et al., 2012). mTOR is an evolutionarily-conserved serine/threonine protein  
75 kinase that senses and integrates a variety of cellular physiological and environmental signals to  
76 regulate cell growth (Jung et al., 2010). The phosphorylated active form of mTOR is involved in  
77 various processes, such as activation of protein translation (transcription, ribosome biogenesis,  
78 protein synthesis) and inhibition of the autophagic activity (Dowling et al., 2010; Soulard et al.,  
79 2009). Despite the large number of studies on mammals demonstrating the existence of multiple  
80 diverse regulators of mTOR and its involvement in the onset of several pathologies (Lapante and  
81 Sabatini, 2012), the research on TOR signalling in invertebrates and in particular in contaminant  
82 exposed organisms is an area where much remains to be explored (Soulard et al., 2009).

83 Molluscs are extensively used as models in many research fields (Abele et al., 2009; Gliński and  
84 Jarosz, 1997); and are widely employed (in particular *Mytilus* sp.) as sentinel organisms in  
85 biomonitoring programs (such as Med Pol, UNEP Mediterranean Biomonitoring Program; OSPAR  
86 Convention; RA.MO.GE.; UNIDO) (Viarengo et al., 2007). The aim of this work was to investigate  
87 the alterations of the lysosomal vacuolar system and the possible involvement of mTOR in their  
88 regulation in the digestive gland of mussels *M. galloprovincialis* Lam. exposed to benzo[a]pyrene  
89 (B[a]P), chosen as model organic xenobiotic. This toxic and genotoxic compound, priority pollutant  
90 listed by U.S. EPA (Environmental Protection Agency) (U.S. EPA, 2009), is ubiquitous in the  
91 environment and tends to persist and bioaccumulate through the food chain (Wang and Wang,  
92 2006).

93 Following exposure to B[a]P, we investigated in mussel digestive gland (organ with storage and  
94 distribution function; Bayne, 2009) firstly the accumulation and the subcellular distribution of  
95 B[a]P, detected by immunofluorescence analysis using an anti-PAHs antibody. Moreover, in this  
96 tissue, the effects on LMS and lysosomal/cytoplasmic (L/C) volume ratio, able to highlight the level

97 of stress in the organisms, from the early warning cellular signals (i.e. increased lysosomal  
98 autophagic activity) to tissue pathology (i.e. excessive autophagy can trigger cell catabolism leading  
99 to a loss of tissue functionality) were also measured. As a possible key element involved in the  
100 regulation of the lysosomal activity, the role of the mTOR was evaluated by immunolabelling. The  
101 level of mTOR phosphorylation was also investigated in mussels sampled in field from areas at  
102 different levels of organic xenobiotic contamination.

103 Mathematical models provide the conceptual and mathematical formalism to integrate molecular,  
104 cellular and whole animal processes (Allen & McVeigh, 2004; Allen & Moore, 2004; Moore &  
105 Noble, 2004). Previous studies have shown that network complexity (as evaluated using network  
106 connectedness -connectance CV%- and node size) can be used as an indicator of homeostasis or  
107 health in cellular systems (Moore, 2010).

108 Modelling is essential for the derivation of explanatory frameworks that facilitates the development  
109 of a predictive capacity for estimating outcomes or risk associated with particular disease processes  
110 and stressful treatments (Moore, 2010; Moore & Noble, 2004; Moore et al., 2015). Previous studies  
111 on mussels and earthworms have shown that there is a strong relationship between lysosomal  
112 membrane stability (LMS), as an indicator of cellular health, and the responses of numerous stress  
113 biomarkers (Moore et al., 2006a; Sforzini et al., 2015, 2017). In this investigation, principal  
114 component analysis and network modelling was used to integrate multi-biomarker data; and to test a  
115 predictive complexity model of cellular patho-physiological function.

116

## 117 **2. Materials and methods**

### 118 *2.1. Chemicals and organisms*

119 All chemicals were of analytical grade and purchased from Sigma-Aldrich Co. (UK/Italy), unless  
120 otherwise indicated. Adult *Mytilus galloprovincialis* Lam. ( $50.7 \pm 2.8$  mm) were collected from the  
121 intertidal zone at Trebarwith Strand, Cornwall, UK ( $50^{\circ} 38' 40''$  N,  $4^{\circ} 45' 44''$  W) in October 2014

122 (Banni et al, 2017). The site is relatively free of disease and is remotely located (Bignell et al.,  
123 2011). Mussels were transported back to the laboratory in cool boxes and allowed to depurate for 7  
124 days in natural seawater from Plymouth Sound. The seawater was maintained at  $15.3 \pm 0.68$  °C and  
125 filtered before to start the experiment ( $\text{pH } 7.9 \pm 0.06$ ). During the depuration period, mussels were  
126 fed with a suspension of *Isochrysis galbana* every 3 days ( $1.05 \times 10^6$  cells/mL), with a 100% water  
127 change 2 h after each feeding.

128

## 129 2.2. *Experimental design and sampling*

130 After depuration, the mussels were transferred to 2-L glass beakers containing 1.8 L of the same  
131 seawater as above and allowed to acclimatize for 48 h. The experiment began after this period and  
132 consisted of a 3-day static exposure with no water changes, during which the mussels were not fed.  
133 Two mussels were used per beaker. A photoperiod of 12 h light : 12 h dark was maintained  
134 throughout the experiment. Good seawater oxygenation was provided by a bubbling system.  
135 Seawater quality was monitored in each of the beakers by measuring salinity ( $35.4 \pm 0.09\text{‰}$ ), pH  
136 ( $7.9 \pm 0.06$ ), % dissolved oxygen ( $97.9 \pm 3.22\%$ ) and temperature ( $15.3 \pm 0.68$  °C) (Banni et al.,  
137 2017). Groups of mussels were exposed to four treatments i.e. solvent control (0.02% dimethyl  
138 sulfoxide [DMSO]; 36 mussels); 5 µg/L B[a]P (36 mussels); 50 µg/L B[a]P (36 mussels); 100 µg/L  
139 B[a]P (36 mussels). After 3 days exposure period, digestive glands were rapidly removed, placed  
140 on aluminium cryostat chucks, chilled in super-cooled n-hexane and stored at -80 °C.  
141 The B[a]P concentrations used in these experiments were selected taking into account that the levels  
142 of PAHs in the sea water of contaminated environment range from 0.26 µg/L (Manodori et al.,  
143 2006), 18.34 µg/L (Sinaei and Mashinchian, 2014) to 46 µg/L (Nasher et al., 2013). After 3 d of  
144 exposure, the amount of chemical in the tissues of exposed animals was similar to that detected in  
145 the tissues of mussels sampled in field contaminated coastal waters (Banni et al., 2017; Widdows et  
146 al., 2002).

147

### 148 *2.3. Lysosomal alterations*

149 Frozen digestive gland sections (10 µm) of mussels from each exposure condition were cut by  
150 cryostat (LeicaCM3050) and flash-dried by transferring them onto slides at room temperature.

151 Lysosomal membrane stability: The determination of LMS in the cells of the digestive gland was  
152 performed on cryostat tissue sections following essentially the method described by Moore (1988).  
153 This cytochemical assay is based on acid labilization characteristics of latent hydrolase  $\beta$ -N-  
154 acetylhexosaminidase (NAH) using naphthol AS-BI-N-acetyl- $\beta$ -D glucosaminide as a substrate for  
155 NAH. Slides were observed using an inverted microscope (Zeiss Axiovert 100M) at 400 ×  
156 magnification, connected to a digital camera (Zeiss AxioCam). The pictures obtained were analysed  
157 using an image analysis system (Scion Image) that allowed for the determination of the labilisation  
158 period i.e. the incubation time in the acid buffer needed to produce the maximal lysosomal staining.

159 Lysosomal/cytoplasmic (L/C) volume ratio: the L/C volume ratio of the digestive gland tissue was  
160 evaluated following the method described by Moore (1976) and Moore and Clarke (1982).

161 Lysosomes were reacted for the lysosomal enzyme  $\beta$ -N-acetylhexosaminidase (NAH) using  
162 naphthol AS-BI-N-acetyl- $\beta$ -D glucosaminide as a substrate for NAH. The ratio between  
163 cytoplasmic and lysosomal volumes was determined by analysing the images obtained from the  
164 slides at 400 × magnification by image analysis as described above and expressed as a percentage  
165 variation with respect to controls.

166

### 167 *2.4. Immunofluorescence analysis*

168 Cryostat frozen digestive gland sections (10 µm) obtained as described above were flash-dried by  
169 transferring them onto poly-L-lysine-coated microscope slides at room temperature and fixed in  
170 paraformaldehyde (PFA) solution (4% PFA in phosphate buffer saline-PBS, pH 7.2, 20 min at 20 ±  
171 1 °C).



172 Immunofluorescent anti-PAHs staining was carried out as described by Sforzini et al., 2014.  
 173 Briefly, after fixation, sections were washed three times in PBS (5 min) and incubated in a  
 174 permeabilisation and blocking solution (0.5% Triton X-100, 2% bovine serum albumin-BSA, 0.5%  
 175 rabbit serum in PBS) for 1 h at  $20 \pm 1$  °C. After rinsing, sections were incubated with the primary  
 176 antibody (monoclonal mouse anti-PAHs, Santa Cruz Biotechnology Inc., 1/100 in PBS containing  
 177 1% BSA and 0.05% Triton X-100) overnight at 4 °C in a moist chamber. Then, the sections were  
 178 washed (three times in PBS, 5 min) and the secondary antibody was applied i.e. polyclonal rabbit to  
 179 mouse IgG (FITC) (Abcam) (1/100 in 1% BSA and 0.05% Triton X-100 in PBS) for 1 h at  $20 \pm 1$   
 180 °C in the dark. Sections were then rinsed in PBS, stained with DAPI (DNA-specific fluorescent  
 181 probe) and then mounted in Mowiol mounting medium (Cold Spring Harb Protoc, 2006).  
 182 Immunofluorescence colocalization of B[a]P and the lysosomal enzyme cathepsin D: following  
 183 immunolabelling with the first primary and secondary antibodies (as described above for single  
 184 labelling), sections were incubated for 1h at RT in PBS containing 2% BSA and 0.5% goat serum  
 185 (Sforzini et al., 2014). Hence, sections were incubated for 2h at 4°C with the second primary  
 186 antibody (rabbit polyclonal to cathepsin D (Abcam) 1/100 in PBS containing 1% BSA) and then,  
 187 after rinsing, in the secondary goat polyclonal to rabbit antibody (DyLight® 594, Abcam, 1/100 in  
 188 1% BSA in PBS, 1h,  $20 \pm 1$ °C) in the dark. Finally, sections were then rinsed in PBS, stained with  
 189 DAPI and then mounted.  
 190 Immunofluorescent anti-mTOR phospho staining: sections prepared as described above were  
 191 incubated in a permeabilisation and blocking solution (0.5% Triton X-100, 2% BSA, 0.5% goat  
 192 serum in PBS, 1 h at  $20 \pm 1$  °C) and then with the primary antibody (anti m-TOR (phospho S2448)  
 193 antibody, Abcam, 1/100 in PBS containing 1% BSA and 0.05% Triton X-100) overnight at 4 °C in  
 194 a moist chamber. Sections were then washed three times in PBS (5 min) and the secondary antibody  
 195 was applied, i.e. polyclonal goat to rabbit (Chromeo) (Abcam) (1/100 in 1% BSA and 0.05% Triton

196 X-100 in PBS) for 1 h at  $20 \pm 1$  °C in the dark. Finally, sections were rinsed in PBS, counterstained  
197 with propidium iodide and mounted.

198 Immunofluorescent anti-mTOR staining: sections were incubated in a permeabilisation and  
199 blocking solution as described above for the anti-mTOR phospho staining. Then, the primary  
200 antibody (anti m-TOR antibody , Abcam, 1/200 in PBS containing 1% BSA and 0.05% Triton X-  
201 100) was applied (overnight at 4 °C in a moist chamber). After washing in PBS, sections were  
202 incubated with the secondary antibody, i.e. goat polyclonal to rabbit antibody (DyLight® 594)  
203 (Abcam) (1/200 in 1% BSA and 0.05% Triton X-100 in PBS) for 1 h at  $20 \pm 1$  °C in the dark.  
204 Finally, sections were rinsed in PBS, counterstained with DAPI and mounted.

205 Controls for non-specific staining included sections that were processed in the absence of the  
206 primary or secondary antibodies: no positive fluorescent stain was observed. Slides were viewed  
207 under  $400 \times$  magnification by an inverted photo-microscope (Zeiss Axiovert 100M connected to a  
208 digital camera Zeiss AxioCam MRm) equipped for fluorescence microscopy using FITC,  
209 Rhodamine and DAPI emission filters. Images were analysed using an image analysis system  
210 (Scion Image) that allowed for the quantification of the mean fluorescence intensity. Sections  
211 double immunolabelled for B[a]P and cathepsin D were viewed under  $400 \times$  magnification by Axio  
212 Observer and images were taken with ApoTome.2 (Zeiss, Germany).

213

#### 214 2.5. Sardinia samples and sampling sites: Field study

215 Mussels (*M. galloprovincialis* Lam.), 4-5 cm in length, were obtained from a farm in Arborea (OR,  
216 Sardinia, Italy) and kept in cages (240 mussels per site splitted in five bags) for 28 days (October-  
217 November 2013) at three sites along the Sardinian coast: Porto Mannu li Fornelli ( $40^{\circ}59'32.1''\text{N}$   
218  $8^{\circ}12'54.5''\text{E}$  -reference site), Cala Reale ( $41^{\circ}03'42.7''\text{N}$   $8^{\circ}17'17.5''\text{E}$  -a small marina), and Porto  
219 Torres ( $40^{\circ}50'23.1''\text{N}$   $8^{\circ}24'16.9''\text{E}$  -large industrial and commercial seaport). Mussels were caged in  
220 polypropylene mesh bags placed about 4 m under the sea surface. At the end of the period of

221 caging, mussel digestive glands were excised, placed on aluminium cryostat chucks, chilled in  
222 super-cooled n-hexane and stored at -80 °C. A large number of biomarkers have been measured in  
223 digestive glands of mussels from the three sites; in this study, we investigated in these tissues the  
224 response of mTOR.

225

## 226 2.6. *Univariate statistical analysis*

227 For B[a]P experiment, at least five replicates per control and per concentration were analysed. Each  
228 replicate consists of the digestive gland from one mussel; the mussels were collected from a  
229 separate beaker. For the field study, at least five replicates per caging site were analysed. Each  
230 replicate consists of the digestive gland from one mussel; the mussels were collected from five  
231 bags. The non-parametric Mann-Whitney *U*-test was used to compare the data from treated mussels  
232 with those of the controls ones.

233

## 234 2.7. *Multivariate statistical analysis*

235 Biomarker data for mussels exposed to B[a]P were analysed using non-parametric multivariate  
236 analysis software, PRIMER v 6 (PRIMER-E Ltd., Plymouth, UK; Clarke, 1999; Clarke &  
237 Warwick, 2001). All data were log transformed [ $\log_n(1+x)$ ] and standardised to the same scale.  
238 Principal component analysis (PCA) and hierarchical cluster analysis, derived from Euclidean  
239 distance similarity matrices were used to visualise dissimilarities between sample groups. The  
240 results were further tested for significance using analysis of similarity (PRIMER v6 - ANOSIM),  
241 which is an approximate analogue of the univariate ANOVA and reflects on differences between  
242 treatment groups in contrast to differences among replicates within samples (the *R* statistic). Under  
243 the null hypothesis  $H_0$  (“no difference between samples”),  $R = 0$  and this was tested by a non-  
244 parametric permutations approach; there should be little or no effect on the average *R* value if the  
245 labels identifying which replicates belong to which samples are randomly rearranged.

246 Finally, in order to map integrated biomarker data onto “health status space” (measured as system  
247 complexity - connectance  $C_v\%$ ) first principal components (PC1) for the biomarker data were  
248 derived using PRIMER v6 and then plotted against the complexity values (as a measure of cellular  
249 well-being) for each treatment (Allen and Moore, 2004; Moore et al., 2006a; Sforzini et al., 2015,  
250 2017).

251

## 252 *2.8. Network modelling of biomarker data*

### 253 *2.8.1. Model description*

254 The generic cell model described by Moore (2010) has been developed from extensive published  
255 data in the environmental toxicology and biomedical literature, and the large-scale organisation of  
256 metabolic networks (Cuervo, 2004; Di Giulio & Hinton, 2008; Jeong et al., 2000; Klionsky & Emr,  
257 2000). The generic cellular interaction network was constructed around the essential processes of  
258 feeding, excretion and energy metabolism. Protein synthesis and degradation, including lysosomal  
259 autophagy, are also incorporated in the model as are the major protective systems (Cuervo, 2004; Di  
260 Giulio & Hinton, 2008; Livingstone et al., 2000; Moore, 2008; Moore et al., 2015). A modified  
261 subset of the generic model was used in this investigation in order to accommodate the available  
262 data (Fig. 8). The directed cellular physiological networks were constructed using Cytoscape 3.3.0  
263 (Shannon et al., 2003).

264

### 265 *2.8.2. Analysis of cell system complexity*

266 Whole system complexity in the directed cellular physiological network was evaluated using  
267 connectedness (Bonchev, 2003). Topological complexity was measured as connectedness or  
268 connectance ( $C_v\%$ ) is the ratio between the number of links  $E$  in the interaction network and the  
269 number of links in the complete graph having the same number of nodes or vertices ( $V$ ) (Bonchev,  
270 2003). Connectedness relates the number of nodes (vertices)  $V$  and links or edges (arcs in a directed

link)  $E$  where the connectance ratio,  $C_V$ , of a directed graph (digraph) with  $V$  nodes or vertices is then:

$$C_V = [(1 / \max(CV)) / \|E\|] \times 100$$

which reduces to:  $CV = (\|E\| / V^2) \times 100$

for typical digraphs that allow every node to connect to every other node, where  $\|E\|$  is the nearest integer function of  $E$  (Davis, 1997). This method uses the sum of the edge weights rather than the edge count and allows for self-loops or arcs as with the autophagy process (Fig. 8).

Transformed biomarker data were used to attribute proportional weight values to the interactions (edges) between cellular physiological processes (nodes) as shown in Table 1; and to the nodes, as node size (Fig. 8). The various biomarker mean values were standardised to a proportion of Control values. These standardised biomarker values ( $x$ ) were used for biomarkers that normally decrease with pathology (e.g., lysosomal membrane stability & mTORC1), while biomarkers that normally increase with pathology (e.g., neutral lipid, lysosomal/cytoplasmic volume ratio & lipofuscin) were further transformed to ( $x^{-1}$ ). These values were normalised using  $\log_{10}$  transformation and then inputted as the weight values for the network interactions (edges/links). The standardised biomarker values were used to set node size for comparisons of network topology (see Fig. 8). The Kruskal-Wallis test were applied to the proportional edge (interaction) values of the treatment groups.

### 3. Results

#### 3.1. Cytochemical and immunohistochemical analysis

The concentrations of B[a]P utilised in this study, after 3 d of exposure, did not provoke any effect on vitality of mussels (data not shown).

Immunofluorescence labelling of digestive glands of B[a]P exposed mussel with the anti-PAHs antibody was positive (Fig. 1B-D); no immunopositivity was detected in control animals (Fig. 1A).

Double immunolabelling of sections with antibodies against PAHs and cathepsin D demonstrated

296 that B[a]P accumulated inside lysosomes (Fig. 1F). Quantification of the B[a]P fluorescence signal  
297 by digital imaging (Fig. 1E) showed a significant increase in fluorescence intensity in animals  
298 exposed to all the experimental conditions, with respect to controls; however, the most intense  
299 staining was found at the lower B[a]P concentration (5 µg/L). The examination of unstained serial  
300 sections of B[a]P exposed mussels under UV light highlighted the presence of numerous white-blue  
301 fluorescent droplets; the fluorescence was minimal in the digestive glands of mussels exposed to 5  
302 µg/L and increased from 50 µg/L to 100 µg/L B[a]P (Fig. 2).

303 B[a]P accumulated in the digestive glands of exposed mussels provoked significant alterations to  
304 the lysosomal vacuolar system (Fig. 3). As shown in Fig. 3A, a decrease of LMS was observed at  
305 all the concentrations, that was significant at 50 µg/L and 100 µg/L B[a]P. At the higher B[a]P  
306 concentrations i.e. 50 µg/L and 100 µg/L B[a]P, a significant increase of the lysosomal/cytoplasmic  
307 volume ratio, a biomarker of tissue damage, was also observed (+44% and +42% respectively, with  
308 respect to controls) (Fig. 3B).

309 The use of an anti-mTOR antibody phosphorylated on S2448 revealed in digestive gland sections of  
310 control mussels an immunopositive reaction; in particular, the fluorescence signal was mainly  
311 located in the perinuclear region of the tubule epithelial cells (Fig. 4A). The immunohistochemical  
312 data demonstrated that in the digestive gland cells of mussels exposed to all the different B[a]P  
313 concentrations, the level of phosphorylated mTOR significantly decreased (Fig. 4F); stronger  
314 effects were observed at 50 µg/L B[a]P and in particular at 100 µg/L B[a]P (Fig. 4C-E, F). The  
315 specificity of this mTOR antibody within the mussel digestive gland was demonstrated by western  
316 blot analysis; these results also confirm the dephosphorylation of the protein in B[a]P exposed  
317 mussels (see Supplementary Information for the details of the method and western blot figure -Fig.  
318 S1).

319 The results reported in Fig. 5 clearly demonstrate that mTOR protein level showed a strong increase  
320 in the cytoplasm of the animals exposed to B[a]P, reaching the highest values in the digestive gland  
321 of mussels treated with B[a]P 50-100 µg/L.

322 When the immunofluorescence staining with the anti-mTOR phosphorylated antibody was  
323 performed in digestive gland sections of mussels caged along the Sardinian coast, the analysis  
324 revealed a strong inactivation of mTOR in Porto Torres (a polluted areas) with respect to the  
325 reference site (Porto Mannu li Fornelli) (Fig. 6A, C).

326

### 327 *3.2. Multivariate analysis of biomarker reactions*

328 Principal component (PCA) and hierarchical cluster analysis of all the biomarker reactions showed  
329 that B[a]P had a detrimental effect on the digestive cells of mussels (Fig. 7). Analysis of similarity  
330 shows that these clusters were significantly different (ANOSIM, R Statistic:  $R = 0.856$ ,  $P = 0.001$ ).  
331 Treatments were clearly separated ( $P < 0.01$ ) with the exceptions of the 50 and 100 µg/L, which  
332 overlapped but were still significantly different ( $P < 0.05$ ). Multiple regression analysis of the  
333 biomarker data indicated that all of the biological parameters were significantly correlated ( $P <$   
334  $0.01$ ).

335

### 336 *3.3. Network modelling biomarker reactions to B[a]P treatment*

337 Inputting the biomarker data into the directed cellular interaction network (digraph) model (Fig. 8)  
338 allowed the determination of the system complexity. Complexity values as connectance ratio (Cv%)  
339 for the experimental treatments are shown in Fig. 8, with a considerable significant loss in  
340 connectivity in the B[a]P treated conditions compared with the controls ( $P = 5.52 \times 10^{-8}$ , Kruskal-  
341 Wallis test).

342 The control and treated network topologies differ in node size and are significantly different as  
343 demonstrated by the ANOSIM and PCA/cluster analysis (Fig. 7). The determination of node degree

344 indicated that autophagy was the most highly connected node with 8 degrees (i.e., summation of 3  
345 out-arc, 4 in-arcs and 1 loop), making it an important physiological hub (Fig. 8). System complexity  
346 (connectance %) was strongly correlated with the concentration of B[a]P (inverse), lysosomal  
347 stability (direct), first principal component (direct) and DNA damage (inverse; COMET assay data  
348 from Banni et al, 2017; see also Canova et al, 1998) as shown in Fig. 9.

349

#### 350 **4. Discussion**

351 In this study, we investigated the reactions of the lysosomal vacuolar system and the possible  
352 involvement of mTOR in their regulation in the digestive gland of mussels *M. galloprovincialis*.  
353 exposed to B[a]P. The digestive gland of bivalve mollusks is the principal site of digestion and  
354 absorption (Bayne, 2009); and represents the major tissue involved in the accumulation and  
355 detoxification of organic and inorganic environmental contaminants (Banni et al., 2016; Gomes et  
356 al., 2012; Moore, et al., 2007; Viarengo et al., 1981). The lysosomal vacuolar system of the  
357 digestive gland cells is well-developed, providing for most of the above mentioned functions  
358 (Dimitriadis et al., 2004; Moore, 1988).

359 B[a]P accumulated in digestive tubule epithelial cells of exposed mussels, as revealed by  
360 immunohistochemical analysis of digestive gland tissue sections by using an anti-PAHs antibody.  
361 This method, recently developed in earthworm tissues (Sforzini et al., 2014), proved to be a reliable  
362 tool for demonstrating the presence and the cellular distribution of B[a]P in animals exposed to  
363 soils contaminated by even a minimal amount of this chemical (0.1 ppm). In particular, the use of  
364 the double immunohistochemical method for co-localization of B[a]P and the cathepsin D (a  
365 lysosomal protease highly conserved throughout lower and higher eukaryotes - Phillips et al., 2006)  
366 demonstrated the lysosomal accumulation of the organic xenobiotic compound.

367 Interestingly, the fluorescence intensity was more intense in the digestive glands from mussels  
368 exposed to the lowest B[a]P concentration (5 µg/L). Previous studies demonstrated that different



PAHs such as B[a]P and fluoranthene, when present in the tissues at high concentrations, are progressively compartmentalised in lipid rich vesicles, with fluorescence properties (fluorescence intensity directly related to the chemical amount) (Allison and Mallucci, 1964; Moore et al., 2007; Plant et al., 1985; Sforzini et al., 2014). These chemicals are highly lipophilic and are known to induce an alteration of fatty acid metabolism (lipidosis) (De Coster and van Larebeke, 2012; Moore et al., 2007); lipids are then internalised into the lysosomes by autophagic uptake (Moore, 1988; Podechard et al., 2009). In mussels exposed to all the different concentrations of B[a]P, a significant increase was observed in lysosomal neutral lipid content of the digestive gland cells (Fig. S2 - see Supplementary Information). The comparison between serial sections of tissues from mussels exposed to B[a]P 50, 100 µg/L stained with Oil-Red O (lipid soluble dye commonly used for the histochemical staining of neutral lipids in cryostat tissue sections -Bayliss High, 1984; Moore, 1988) in bright-field and unstained sections by UV-fluorescence, highlighted that the distribution of the B[a]P fluorescent droplets in the digestive gland cells corresponds to that of the neutral lipid containing vesicles (Fig. 10). Recent studies have reported difficulty in labelling target molecules in lipid droplets by immunofluorescence methods, probably because of the reduced accessibility of these compartments to the antibodies (DiDonato and Brasaemle, 2003; Ohsaki et al., 2005; Sforzini et al., 2014). Chemical results support this hypothesis, showing that the amount of B[a]P accumulated in the digestive gland of exposed mussels increased with increasing dose; in addition, the lipid concentration also increased with increasing exposure concentrations (Banni et al., 2017).

Lysosomes of B[a]P exposed mussels showed relevant perturbations in their activity. LMS was reduced in animals exposed to 5 µg/L B[a]P but stronger effects were observed at 50 and 100 µg/L. Lysosomes are the target for many pollutants (both organic xenobiotics as well toxic metals); the chemicals accumulated in lysosomes may perturb normal function and damage the lysosomal membrane (Moore et al., 2006b; Sforzini et al., 2014; Viarengo et al., 1981). Pathological reactions

394 involving the lysosomal system are also often linked to augmented autophagic sequestration of  
395 cytoplasmic components (e.g. autophagic accumulation of neutral lipids) and the removal of  
396 damaged organelles and proteins (which are degraded in lysosomes) (Glick, et al., 2010; Moore,  
397 2008). In vertebrates, quinone derivatives, produced during B[a]P metabolic processes, generate  
398 reactive oxygen species (ROS) by redox cycling, which oxidatively altered DNA, protein, and  
399 antioxidant enzymes (Kim and Lee, 1997). B[a]P is metabolized predominantly to quinones by  
400 mussel digestive gland microsomes (Livingstone et al., 1988; Stegeman, 1985). Although the  
401 hydrocarbon metabolism in molluscs is slow and the rate-limiting cytochrome P450 may be  
402 responsible for this (Livingstone, 1998), previous studies have demonstrated changes in antioxidant  
403 enzymes and peroxisomal proliferation with exposure of mussels to B[a]P (Livingstone et al., 1990;  
404 Orbea et al., 2002), indicating an enhancement of oxyradical generation. In mussels exposed to all  
405 the different concentration of B[a]P utilised in this study, Banni et al. (2017) demonstrated an  
406 increase of lipofuscin accumulated in lysosomes of digestive gland cells. It is likely that reactive  
407 free radicals contribute to the damaging effects on the lysosomal membrane and build-up of  
408 lipofuscin (end product of oxidative attack on lipids and proteins) (Viarengo, 1989; Moore, 2008;  
409 Winston et al., 1996).

410 In this context, the autophagy may have a protective role in the context of (oxidative) stress through  
411 the degradation and recycling of oxidised proteins and damaged organelles (Cuervo, 2004).  
412 However, an excessive autophagic rate has been shown to have deleterious consequences for  
413 tissue/organism health (Levine and Kroemer, 2008). At the higher B[a]P concentrations, a  
414 significant enhancement of L/C volume ratio was also observed. These data indicate that the  
415 animals are catabolic i.e. the autophagic process is highly stimulated and the catabolism of the  
416 macromolecules is not compensated by protein synthesis. The (oxidative) damage to cellular  
417 components may have contributed to decrease protein synthesis (Viarengo, 1989; Winston et al.,

1996; Moore et al., 2006a). The reduction of the cytoplasm of the cells may lead a loss of their proper functionality with negative consequences on digestive gland physiology.

Overall, these results confirm that digestive cell lysosomes are the targets for toxic chemicals and they are also sites of their accumulation. B[a]P stimulated the lysosomal fatty acid accumulation and at the higher concentrations was stored in these compartments. Moreover, at the higher doses, B[a]P also overstimulates the autophagic process leading to cell catabolism and thus tissue pathology. One of the possible processes that could explain the observed effects is the mTOR signal transduction pathway. mTOR (mechanistic target of rapamycin) is an evolutionarily-conserved serine/threonine protein kinase that represents the central node of a highly conserved signalling network regulating cell growth in response to nutrients, hormones and stresses (Jung et al., 2010). mTOR is found in two functionally distinct complexes, mTORC1 and mTORC2. In particular, the phosphorylated active form of TORC1 mediates temporal control of cell growth by activating anabolic processes such as transcription, ribosome biogenesis, protein synthesis; and by inhibiting catabolic processes such as autophagy (Dowling et al., 2010; Soulard et al., 2009). In mammals a lot of studies have been devoted to investigate mTOR regulators. The dysregulation of mTOR signalling is implicated in a number of human diseases including cancer (Dowling et al., 2010). In invertebrates, the research on TOR signalling, particularly in contaminant exposed organisms is an area where much remains to be explored (Soulard et al., 2009).

Recently, Copp et al. (2009) showed that mTOR is phosphorylated differentially when associated with mTORC1 and mTORC2; specifically, they found that mTORC1 contains mTOR phosphorylated predominantly on S2448. The immunofluorescence labelling of control digestive gland sections using an anti-mTOR antibody phosphorylated on S2448 revealed an immunopositive reaction; in particular, the fluorescence signal was mainly located in the perinuclear region of the tubule epithelial cells. These results are in line with other studies showing that mTORC1 in different kind of cells (such as trypanosomes, yeast and mammalian cells) localizes mainly to the

nucleus (Barquilla et al., 2008; Li et al., 2006). When the digestive gland cells of mussels exposed to all the different B[a]P concentrations were reacted for the anti-mTOR (phospho S2448) antibody, the fluorescent signal decreased; at 50 µg/L B[a]P and in particular at 100 µg/L B[a]P, i.e. concentrations that provoked a sustained increase of the cellular catabolic rate, we observed dramatic changes. Although the mechanisms that regulate the mTOR dephosphorylation are not till now fully understood, the possibility that B[a]P stimulating ROS production may affect mTOR activities is in line with recently reported results (Chen et al., 2010; Moore, 2008). It is important to mention that the total amount of mTOR showed a significant increase in mussels exposed to the different B[a]P concentrations (Fig. 5). This fact emphasises the importance of mTOR phosphorylation/dephosphorylation in the regulation of cell metabolism.

An important aspect of this study was to investigate if the data obtained could have a general value, i.e. if the level of phosphorylation of mTOR (activation/inhibition) could be also observed in mussels exposed to field environmental conditions. To this end, we analysed mussels that were caged for 28d in different sites along the Sardinian coast characterized by different levels of contamination i.e. Fornelli, the reference site, Cala Reale, a small marina, and Porto Torres, large industrial and commercial seaport contaminated by PAHs and heavy metals. An intense mTORC1 fluorescent signal was observed in the perinuclear area of the digestive gland cells of mussels caged in the reference site (Fornelli); immunofluorescence staining showed a decrease in mussels caged in Cala Reale. In mussels caged in Porto Torres, the level of mTOR phosphorylation was extremely low; in the digestive glands of the same animals we have found a strong decrease of LMS and an enhancement of L/C volume ratio; as well oxidative stress damage (Banni et al., manuscript in preparation). The “picture” depicted by the field experiment is very similar to that observed in the lab one.

Principal Component Analysis (PCA) is an effective method for integrating biomarker data into a “health status space” reducing the multi-dimensionality of the problem to a simple two dimensional

468 representation (Chatfield and Collins, 1980; Allen and Moore, 2004). PCA is commonly used as a  
469 cluster analysis tool and effectively captures the variability in a dataset in terms of principal  
470 components, and previously PCA has facilitated modelling the integrated responses of multiple  
471 biomarkers in the context of “health status space” (Allen and Moore, 2004; Moore et al., 2006a).  
472 These models have shown that there is a strong relationship between LMS, as an indicator of  
473 cellular health, and other combined biomarker responses (Allen & Moore, 2004; Moore et al.,  
474 2006a; Sforzini et al., 2015, 2017).

475 However, PCA and cluster analysis does not integrate the various biomarkers in a functionally  
476 meaningful way, and is only the first stage in developing numerical and network models for  
477 environmental impact on the health of sentinel animals such as mussels and earthworms (Allen and  
478 Moore, 2004; Moore, 2010; Sforzini et al., 2015, 2017). In order to encapsulate the cellular  
479 physiological processes, it is necessary to interconnect the biomarker data into a logical framework.  
480 This was done using a network model of the physiological/pathological processes known to occur in  
481 the digestive cells. Complexity is a measure of the interconnectedness of the network and can be  
482 used as an indicator of homeostasis (Lewis et al., 1992; Moore, 2010; Moore et al., 2015; Sedivy,  
483 1999). Complexity of the whole system increases when sub-systems, such as detoxication and anti-  
484 oxidant protective processes, augmented autophagy, protein degradation and induction of stress  
485 proteins, are up-regulated and start to interact significantly as part of a response to low-level stress  
486 (i.e., biphasic or hormetic response; Moore, 2010; Moore et al., 2015). However, with increasing  
487 severity of stress, cell injury and higher-level functional impairment lead to physiological  
488 dysfunction, pathology and breakdown of the whole interaction network with consequent loss of  
489 complexity (Moore, 2010). Consequently, inputting the biomarker data from the B[a]P exposure  
490 experiment into a directed cell physiology network model showed that there was a statistically  
491 significant reduction in system complexity with increasing tissue B[a]P, indicating decreased  
492 homeostasis and health status (Fig. 9; Table 1). Network topology was also significantly different in

terms of node size (Fig. 8). The model demonstrates that autophagy is an important highly connected hub in the cellular physiology of the system being tested, which lends support to the overall hypothesis, namely, that autophagy, lysosomal function and mTOR signaling are intrinsically interlinked in responses/reactions to stress (Fig. 8). The strong correlations between network complexity, B[a]P concentration, lysosomal stability and first principal component further support the use of system complexity as a measure of cellular homeostasis (Fig. 9).

The network approach supports the hypothesis that stress leading to pathology results in a loss of system complexity as previously described by Moore (2010). Consequently, cellular networks can be used to integrate information from biomarker data; and to direct the selection of biomarkers and design of experiments, in order to develop suites of tests that will demonstrate which links are active or inactive, and to what degree. This provides mathematical formalism for an objective evaluation of health status for potential use in risk assessment (Moore, 2002, Moore et al., 2004b).

Cellular interaction networks also have considerable potential for integrating multi-biomarker data for evaluation of whole system “health status” (Moore, 2010). The strong correlation between system complexity and DNA damage indicates that this type of modelling has potential for predicting cellular pathological endpoints (Canova et al., 1998; Fig. 9).

Finally, the network model facilitates the development of a mechanistic framework that encapsulates the interrelated patho-physiological processes that are involved in the cellular reactions to the B[a]P. These processes are described diagrammatically in Figure 11, although all of them are evolutionarily highly conserved, some are not yet confirmed to occur in molluscs (i.e., mTOR links with endocytosis and MDR/Pgp40 multi-drug resistance transporter). This diagram shows the linkages between endocytotic uptake of B[a]P with natural particles, transfer to the lysosomal system, where accumulation will be further facilitated by P-glycoprotein (MDR-Pgp40) in the lysosomal membrane (Minier & Moore, 1996a, b; Yang et al., 2002). Accumulation of B[a]P and lipid in the lysosomal compartment results in ROS generation and formation of lipofuscin

(stress or age pigment) (Brunk & Terman, 2002; Moore et al., 2006a). Oxidative stress may have a positive feedback inhibiting mTORC1 and enhancing autophagy (Brunk & Terman, 2002; Chen et al., 2010; Moore et al., 2006a, 2015). Inhibition of mTORC1 will also inhibit endocytosis, lysosomal membrane stability and activate Pgp40 (Boya, 2012; Flinn & Backer, 2010; Jiang & Liu, 2008). The increased flux of ROS will also contribute to oxidative damage to DNA (Canova et al., 1998); and enhanced autophagy may engulf portions of damaged and undamaged genomic material through partial nuclear autophagy (Buckland-Nicks & Hodgson, 2005; Mochida et al., 2015). Autophagy of nuclear DNA may contribute to autophagic and/or apoptotic cell death as a pathological endpoint (Lowe, 1988); and may be protective against the development of digestive gland tumours, which are extremely rare in molluscs (Khudolei & Sirenko, 1977).

528

## 529 **5. Conclusions**

Overall, the data obtained in this work demonstrate that the signal transduction pathways linked to mTOR (and in particular to mTORC1) could play an important role to determine the set of the pathological effects that render the organisms “catabolic” and therefore no more able to sustain a correct scope for growth. The probable role of mTOR in cell signalling and the regulation of the cellular responses to the contaminants has been confirmed in a field study, where in the digestive gland of mussels sampled from contaminated sites there was an inactivation of mTOR. Obviously, as mentioned above, part of the shown effects (and others such as DNA damage) may depend on the direct effect of the toxic chemical on the different cellular components. The analysis of the data by the network connectedness demonstrates that autophagy, lysosomal function and mTOR signalling are intrinsically interlinked in responses/reactions to stress. This network approach supports the hypothesis that stress leading to pathology results in a loss of system complexity (Moore, 2010). Cellular interaction networks also have considerable potential for integrating multi-biomarker data

542 for evaluation of whole system “health status” and for potential use in risk assessment (Moore,  
543 2010).

544

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548

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 798

799 **Table 1. Network model interactions and corresponding biomarker used for ascribing**  
800 **interaction strength.**

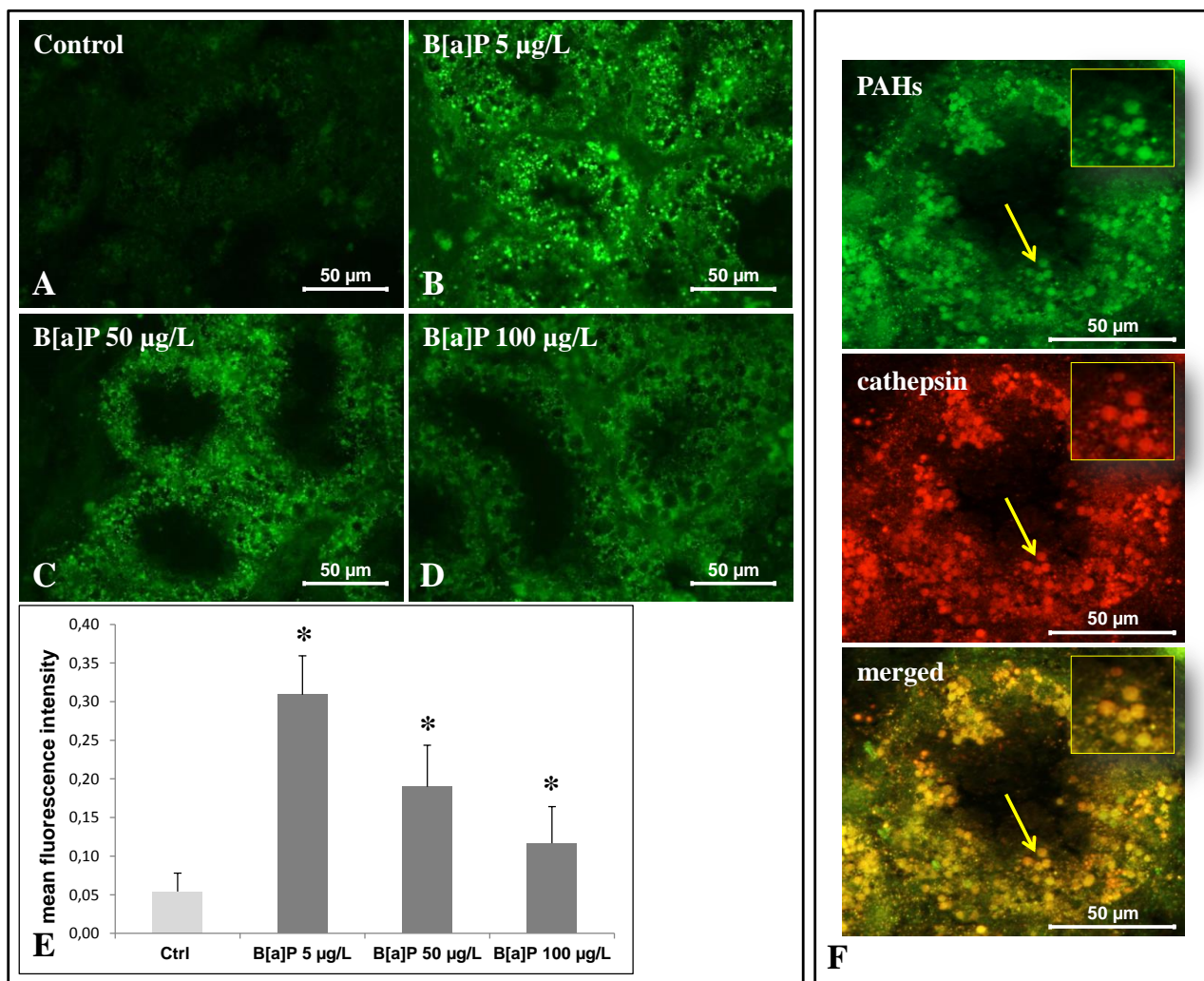
801

<i>Interaction</i>	<i>Biomarker</i>
<b>LYS-AUT</b>	Lysosomal Membrane Stability (LMS)
<b>LYS-OxSt</b>	Lysosomal Membrane Stability (LMS)
<b>AUT-LYS</b>	Lysosomal -Cytoplasmic Volume Ratio (Lys/Cyt Vol)
<b>AUT-OxSt</b>	Lysosomal -Cytoplasmic Volume Ratio (Lys/Cyt Vol)
<b>AUT-AUT</b>	Lysosomal -Cytoplasmic Volume Ratio (Lys/Cyt Vol)
<b>AUT-DNA dam</b>	Lysosomal -Cytoplasmic Volume Ratio (Lys/Cyt Vol)
<b>LIPID-LYS</b>	Neutral Lipid (NL)
<b>LIPID-AUT</b>	Neutral Lipid (NL)
<b>mTORC1-LYS</b>	mTORC1 (mTOR)
<b>mTORC1-AUT</b>	mTORC1 (mTOR)
<b>OxSt-AUT</b>	Lipofuscin (LF)
<b>OxSt-LYS</b>	Lipofuscin (LF)
<b>OxSt-LIPID</b>	Lipofuscin (LF)
<b>OxSt-mTOR</b>	Lipofuscin (LF)
<b>OxSt-DNA dam</b>	Lipofuscin (LF)

802

803 **LYS – Lysosomal function; AUT – Autophagy; OxSt – Oxidative stress; DNA dam – DNA damage;**  
804 **Lipid – Neutral lipid (triglyceride); mTORC1 – Mechanistic target of rapamycin complex 1.**

805



806

807 Fig. 1.

808

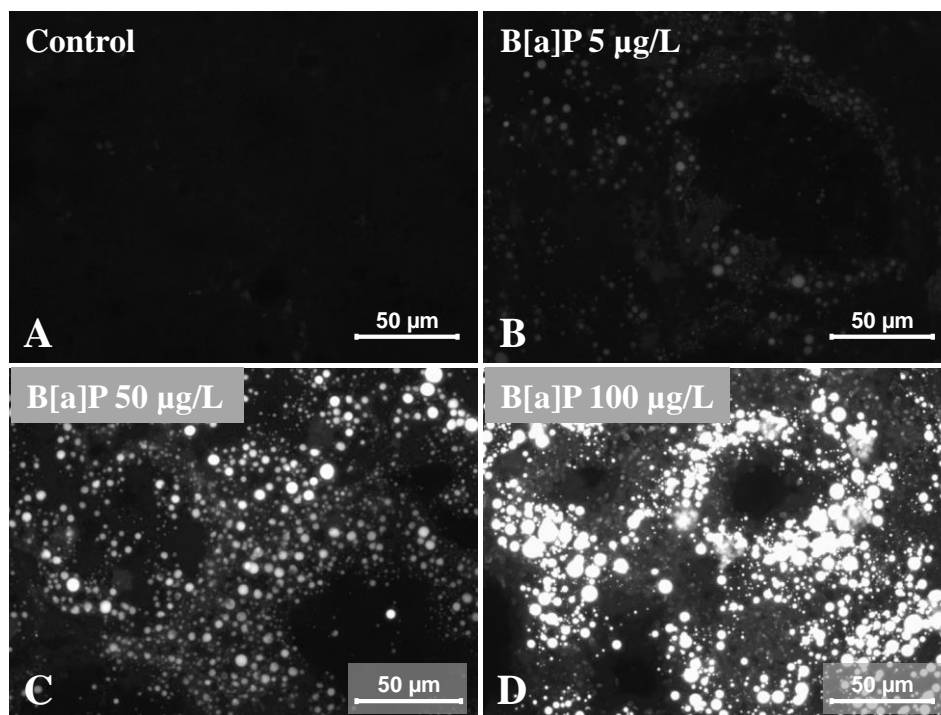


Fig. 2.

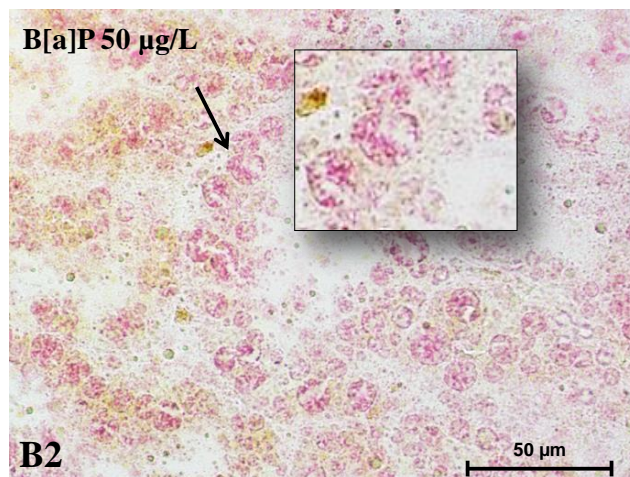
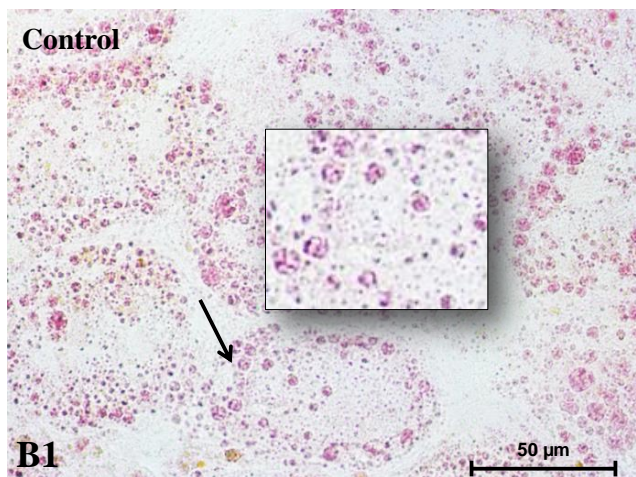
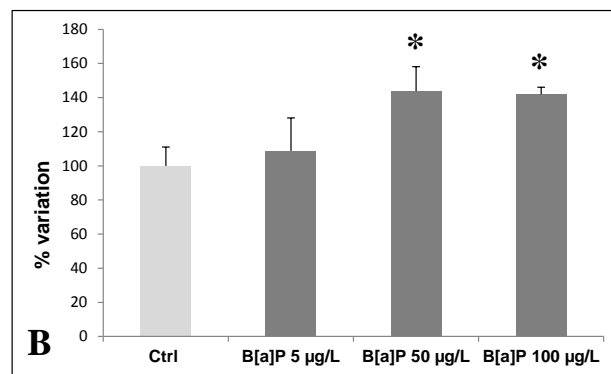
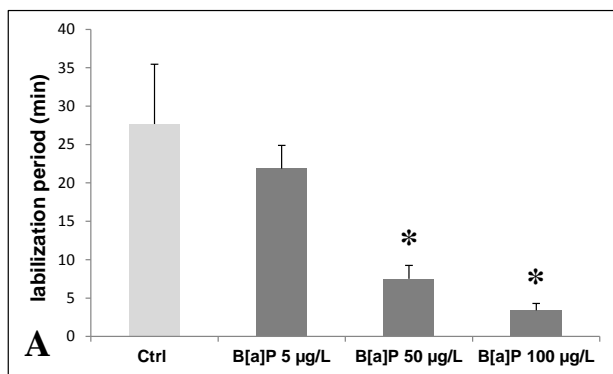


Fig. 3.

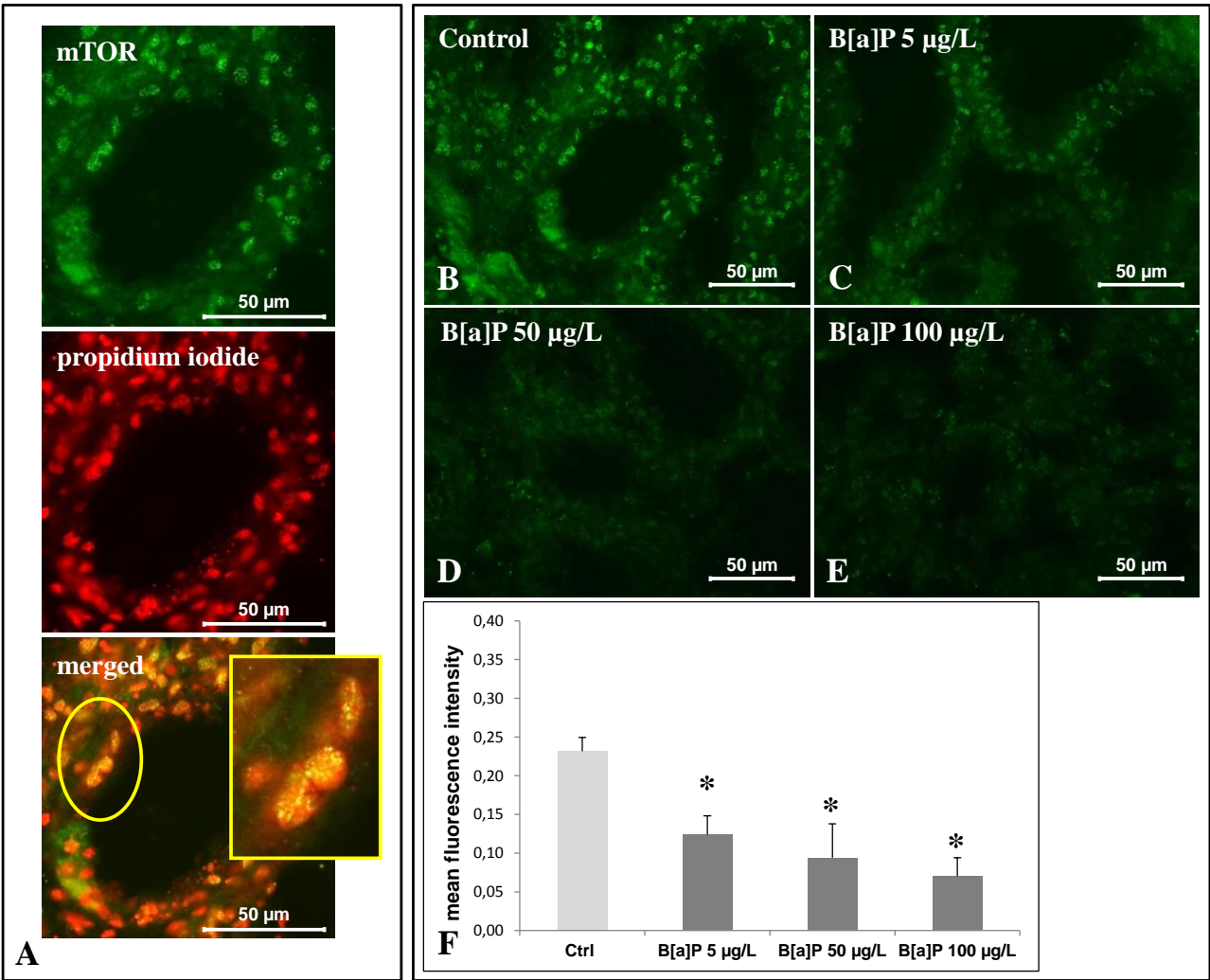
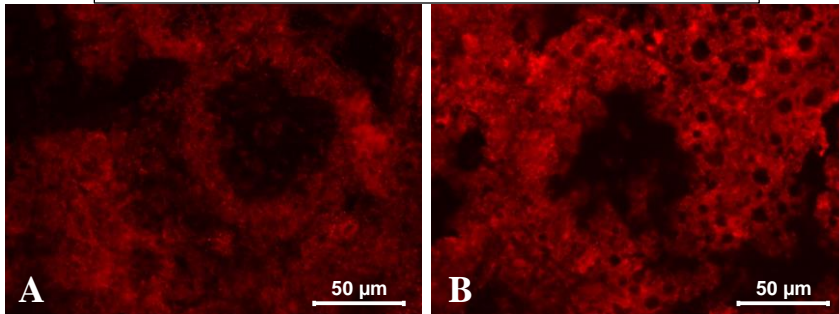
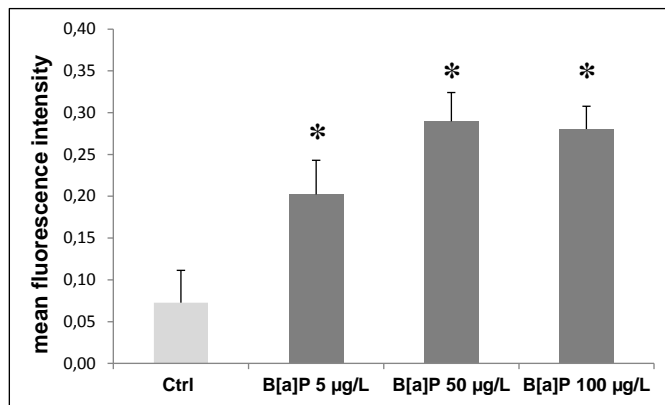


Fig. 4.



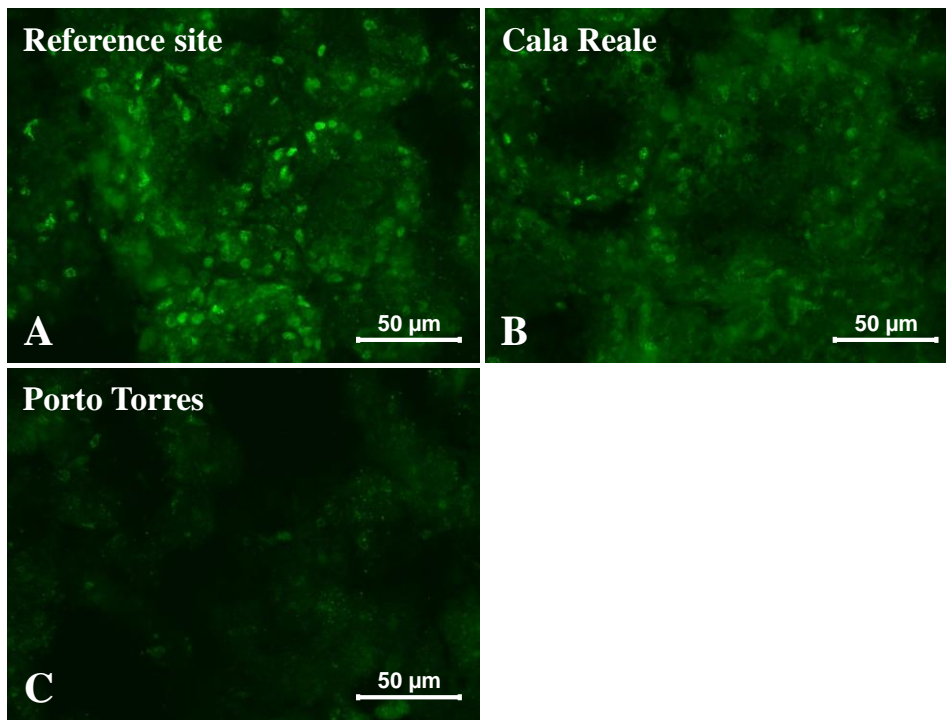


818

819 Fig. 5.

820

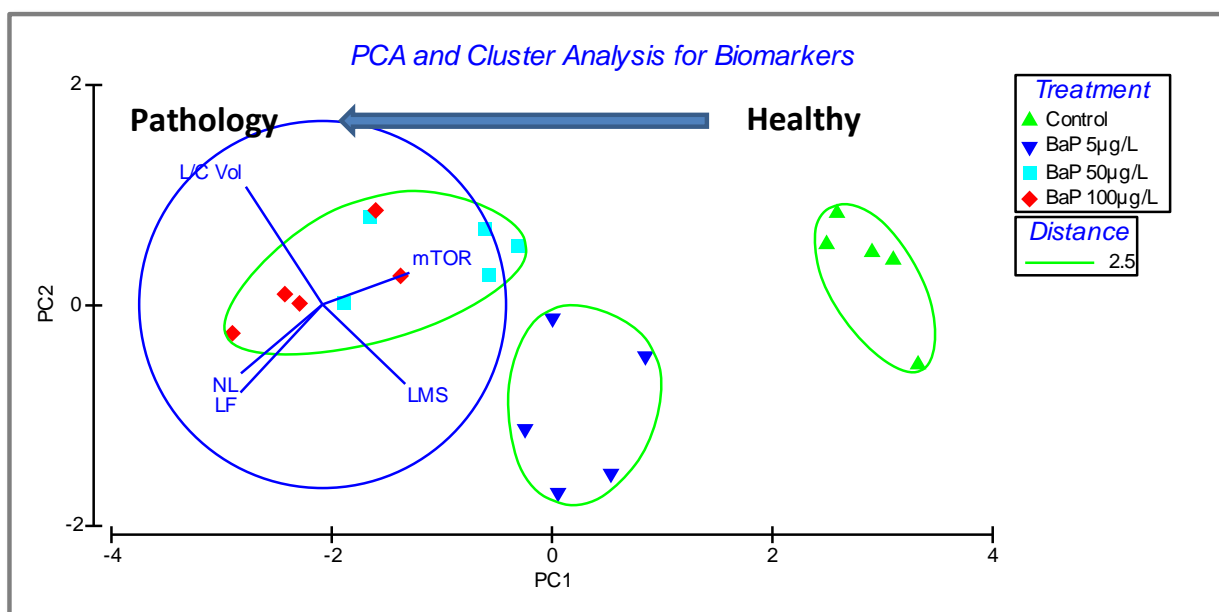




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822 Fig. 6.

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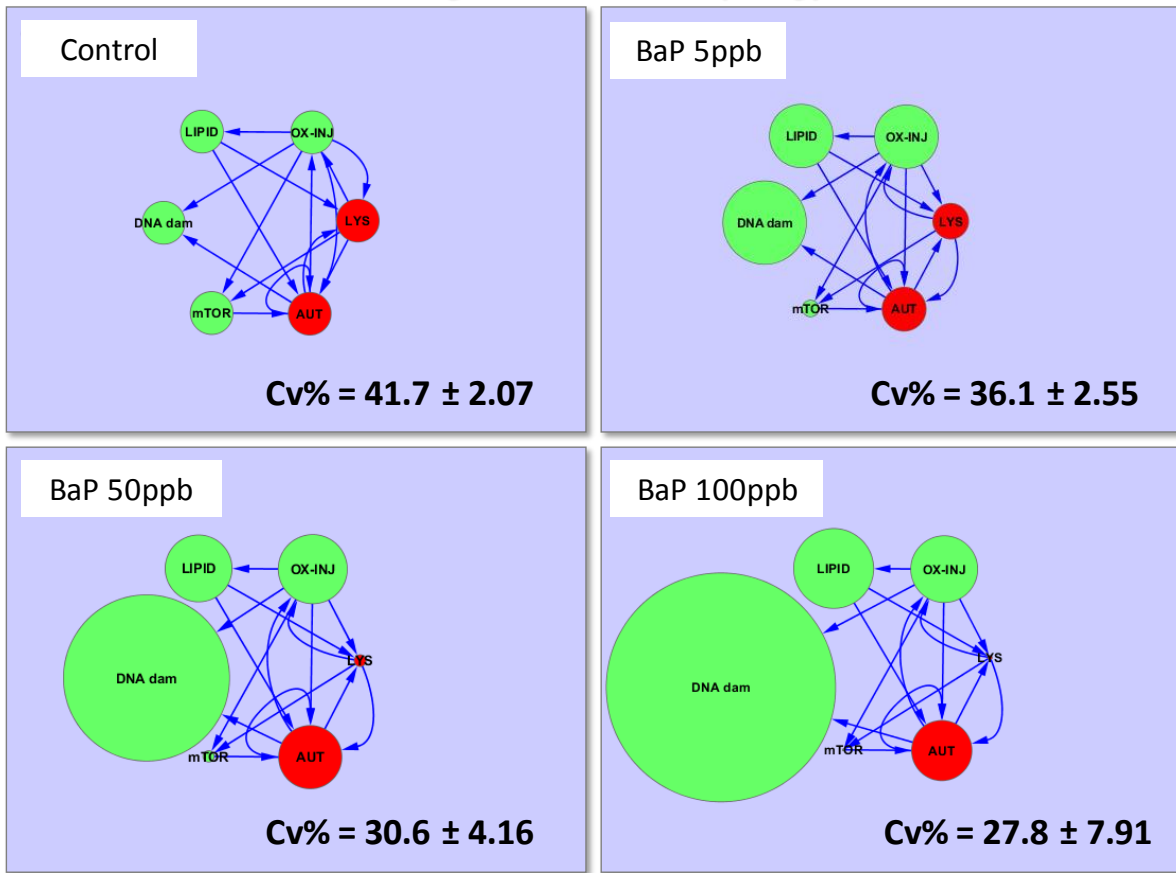


824

825 Fig. 7.

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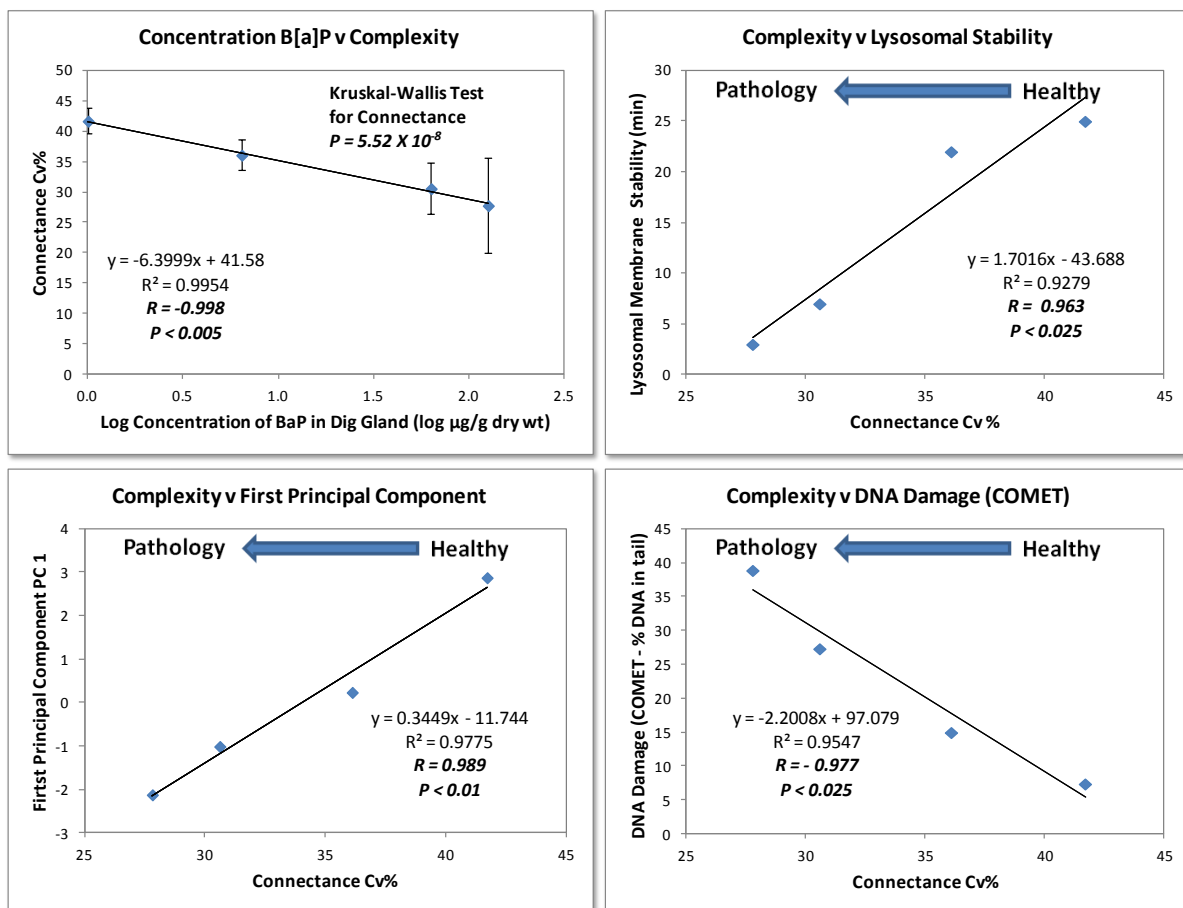
## Changes in Network Topology

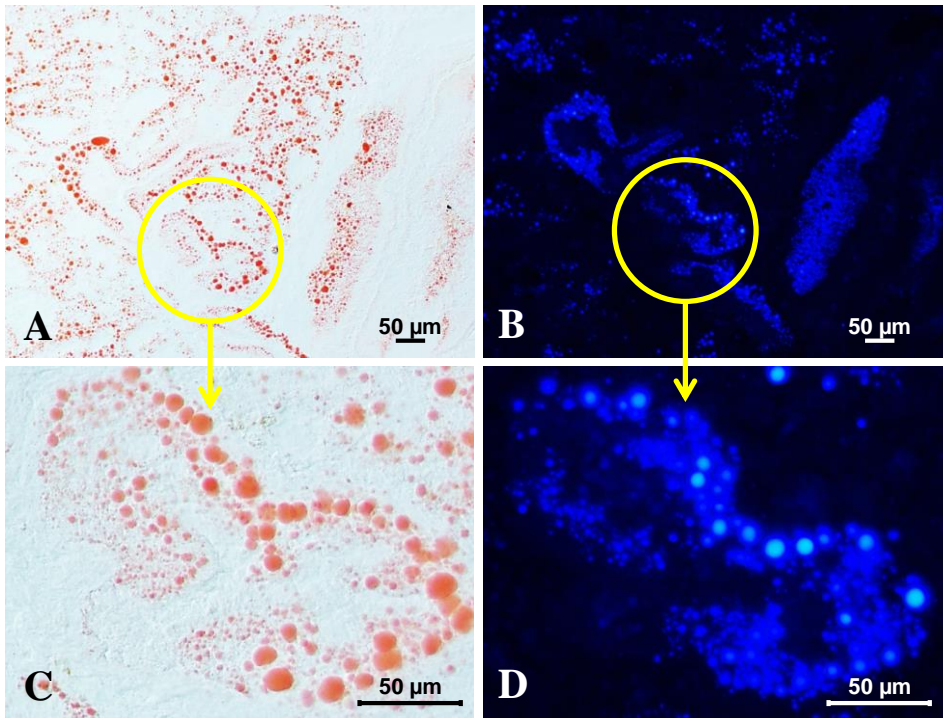


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828 Fig. 8.

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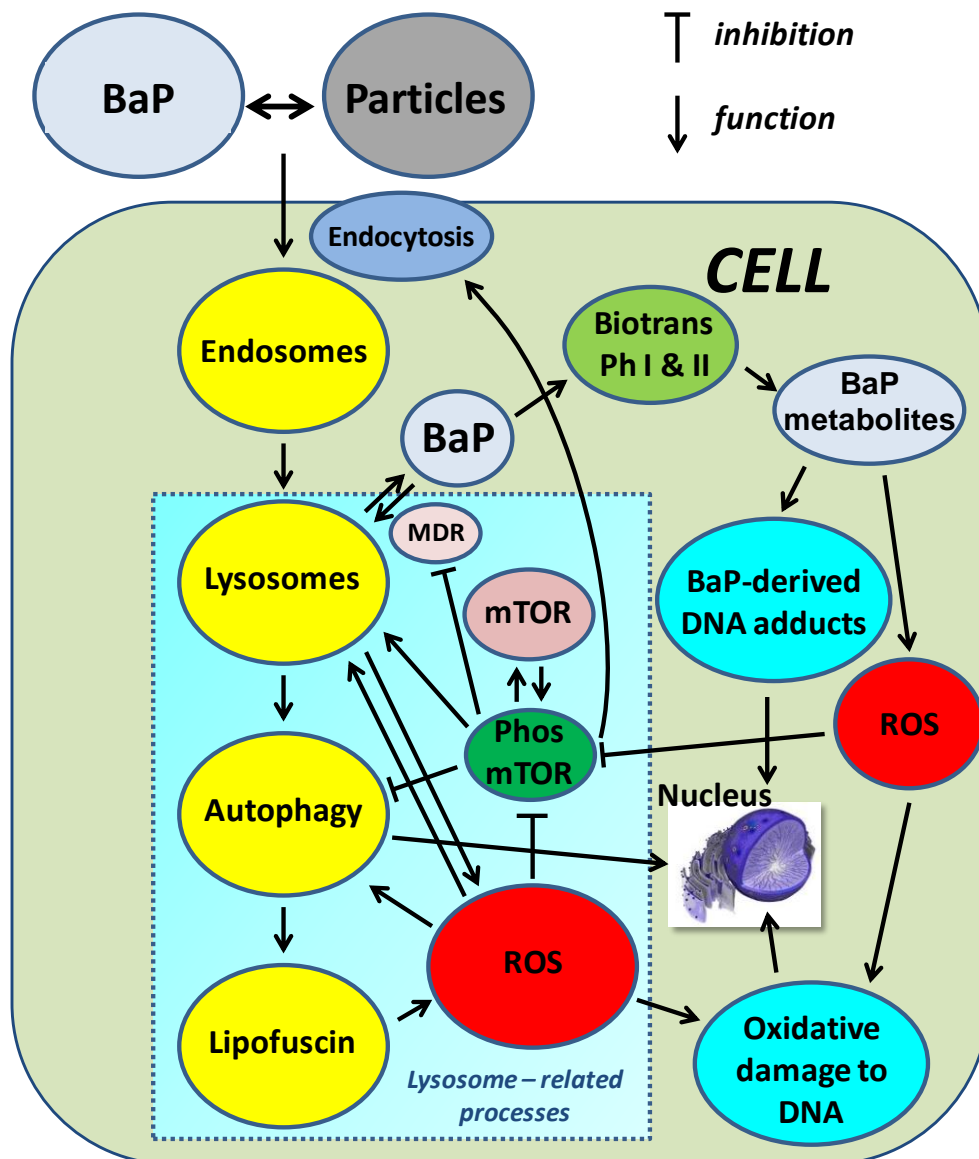


833

834 Fig. 10.

835

## Mechanistic model of BaP uptake, intracellular fate and effects



836

837

838 Fig 11.

839

840 Fig. 1. Anti-PAHs immunohistochemical staining (green: FITC conjugated secondary antibody) of  
841 digestive gland tissue sections from mussels exposed to different experimental conditions (A:  
842 Control; B: 5 µg/L B[a]P; C: 50 µg/L B[a]P; D: 100 µg/L B[a]P). E) Quantitative fluorescence  
843 analysis of anti-PAHs immunoreaction. Data are mean  $\pm$  SD of at least five replicates; \* =  $p < 0.05$   
844 (Mann-Whitney *U*-test). F) Double immunohistochemical staining of digestive glands from mussels  
845 exposed to 5 µg/L B[a]P with anti-PAHs and -cathepsin D antibodies (separate colour images for  
846 PAHs (FITC, green) and cathepsin D (DyLight594, red) immunoreactivity were merged into a  
847 composite image, whereby the colocalization of both antigens in lysosomes of B[a]P exposed  
848 mussels was revealed through the coincidence of the two labels resulting in a yellow colour -see  
849 arrows and insets).

850

851 Fig. 2. Cryostat unstained sections of digestive glands from mussels exposed to different  
852 experimental conditions (A: Control; B: 5 µg/L B[a]P; C: 50 µg/L B[a]P; D: 100 µg/L B[a]P)  
853 examined with UV excitation: white-blue fluorescent deposits, in form of droplets, were evident  
854 particularly at the higher B[a]P concentrations (C, D) (grayscale images).

855

856 Fig. 3. Lysosomal biomarker responses in digestive gland of mussels exposed to B[a]P (5, 50, 100  
857 µg/L). A) Lysosomal membrane stability (cytochemical assay based on acid labilization  
858 characteristics of latent hydrolase  $\beta$ -*N*-acetylhexosaminidase); B) lysosomal/cytoplasmic volume  
859 ratio (lysosomes reacted for the lysosomal enzyme  $\beta$ -*N*-acetylhexosaminidase: when compared to  
860 controls (B1), in mussels exposed to B[a]P an enlargement of autolysosomes was observed (B2),  
861 see arrows and insets). Data represent the mean  $\pm$  SD of at least five replicates. \* indicates  
862 statistically significant differences ( $p < 0.05$  Mann-Whitney *U*-test).

863

864 Fig. 4. Anti-mTOR (phospho S2448) immunohistochemical staining (green: Chromeo conjugated  
865 secondary antibody) of digestive gland tissue sections from mussels exposed to different  
866 experimental conditions. (A; B) Control (in A separate colour images for mTOR immunoreactivity  
867 (Chromeo, green) and the nuclear counterstain propidium iodide (red) were merged into a  
868 composite image, whereby the yellow colour highlights the localization of mTOR in perinuclear  
869 region of the tubule epithelial cells); C) 5 µg/L B[a]P; D) 50 µg/L B[a]P; E) 100 µg/L B[a]P). (F)  
870 Quantitative fluorescence analysis of anti-mTOR immunoreaction. Data are mean  $\pm$  SD of at least  
871 five replicates; \* =  $p < 0.05$  (Mann-Whitney *U*-test).

872  
873 Fig. 5. Quantitative fluorescence analysis of anti-mTOR immunoreaction of digestive gland tissue  
874 sections from mussels exposed to B[a]P. Data represent the mean  $\pm$  SD of at least five replicates. \*  
875 indicates statistically significant differences ( $p < 0.05$  Mann-Whitney *U*-test). Representative  
876 images of tissue sections of controls (A) and 50 µg/L B[a]P exposed mussels (B) (red: DyLight594  
877 conjugated secondary antibody).

878  
879 Fig. 6. Anti-mTOR (phospho S2448) immunohistochemical staining (green: Chromeo conjugated  
880 secondary antibody) of digestive gland tissue sections from mussels caged at three sites along the  
881 Sardinian coast. A) Reference site (Porto Mannu li Fornelli); B) Cala Reale; D) Porto Torres.

882  
883 Fig. 7. Principal component (PCA) and cluster analysis of the biomarker data not including DNA  
884 damage. Vectors indicate the directionality of specific biomarkers.

885  
886 Fig. 8. Interaction network models based on the physiological and pathological processes  
887 represented by the biomarker investigations in mussel digestive cells. Processes represented include  
888 lysosomal function, autophagy, mTORC1 signalling, lysosomal lipid accumulation, oxidative injury



889 and DNA damage. Node sizes are based on the proportional change in the biomarker representing  
890 the process (see Table 1). System complexity (Connectance  $C_v\% \pm 95\% \text{ CL}$ ,  $n = 5$ ) is shown for  
891 each treatment.

892

893 Fig. 9. Statistical modelling for system complexity versus B[a]P concentration (showing  $\pm 95\% \text{ CL}$   
894 for  $C_v\%$ ,  $n = 5$ ), lysosomal stability, first principal component and DNA damage (COMET).

895

896 Fig. 10. Representative images of cryostat serial sections of digestive glands from mussels exposed  
897 to B[a]P 100  $\mu\text{g/L}$  (A, C) stained with Oil-Red O for the evaluation of lysosomal neutral lipid  
898 content and (B, D) unstained and analysed with UV excitation, showing that the distribution of the  
899 B[a]P fluorescent droplets in the digestive gland cells corresponds to that of the neutral lipid  
900 containing vesicles.

901

902 Fig 11. Diagrammatic representation of an explanatory mechanistic framework for the  
903 interconnected cellular reactions to B[a]P based on the biomarker data, network modelling and  
904 other published sources in the scientific literature. ROS - reactive oxygen species; Phos mTOR -  
905 active phosphorylated form of mTORC1 cell signalling system; mTOR - inactive dephosphorylated  
906 form of mTORC1; MDR – Pgp40 multidrug transporter; BIOTRANS Ph I & II - Phase I and II  
907 biotransformation system (Canova et al., 1998).

908

- 909 -The autophagic process in digestive gland of B[a]P exposed mussels was investigated.
- 910 -B[a]P accumulated in lysosomes/enlarged lipid-rich lysosomes of digestive cells.
- 911 -At higher doses B[a]P overstimulated the autophagy and increased cell catabolism.
- 912 -B[a]P-induced dephosphorylation of mTOR may explain the observed pathological effects.
- 913 -Network connectedness showed that pathology results in a loss of system complexity.
- 914

## 915 **Supplementary Information**

916

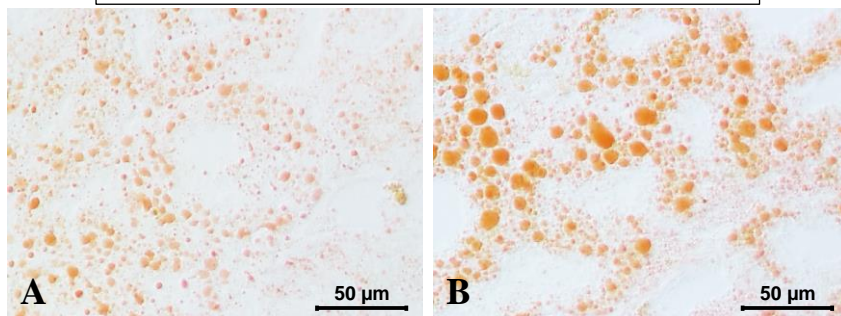
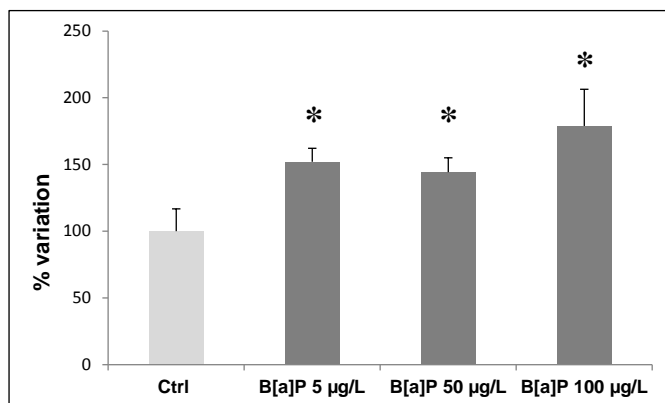
### 917 *Western blot analysis*

918 Digestive glands were homogenised with NP-40 buffer (150mM sodium chloride, 1% Triton,  
 919 50mMTris, pH 8.0) containing 1/100 of protease inhibitor cocktail (Sigma-Aldrich) and centrifuged  
 920 at 1000 rcf for 5 min at 4°C. Pellets were resuspended in the NP-40 buffer and 10 µg of proteins  
 921 were loaded on a Mini-Protean TGX 4-15% gel (Bio-Rad Laboratories S.r.l ) for SDS-PAGE, under  
 922 reducing conditions. Following electrophoresis, the proteins were transferred onto PVDF  
 923 membranes in transfer buffer. The membranes were blocked with 5% BSA solution at 4 °C for 1 h.  
 924 The blots were incubated overnight at 4°C with the primary antibody (anti m-TOR (phospho S2448)  
 925 antibody, Abcam, ab84400) at 1 ug/ml, followed by incubating with a 1:5000-diluted HRP-  
 926 conjugated goat anti-rabbit secondary antibody (Bio-Rad Laboratories S.r.l.) for 90 minutes, and  
 927 then visualized by Clarity™ ECL detection kit. (Bio-Rad Laboratories S.r.l.).  
 928



930 Fig. S1. Western blot analysis of p-mTOR (S2448) protein indicating that B[a]P induces a  
 931 dephosphorylation of the protein. Protein bands shown are representative of 3 independent  
 932 experiments with similar results.

933



934

935 Fig. S2. Lysosomal neutral lipid content in the digestive gland cells of mussels exposed to B[a]P (5,  
 936 50, 100 µg/L). Data, expressed as percent change with respect to control values, represent the mean  
 937  $\pm$  SD of at least five replicates. \* indicates statistically significant differences ( $p < 0.05$  Mann–  
 938 Whitney *U*-test). Representative images of tissue sections from control (A) and B[a]P-exposed  
 939 mussels (B) (100 µg/L).